An Improved Method for Extraction of DNA from Envelopes

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Abstract

At the Armed Forces DNA Identification Laboratory (AFDIL) envelopes can be used as alternative reference samples to help in the human identification process. Currently at AFDIL, envelope DNA extractions are completed by: 1) using a steam bath to open the envelope seal, 2) swabbing a dime-sized portion of the envelope seal to collect buccal cells deposited when the envelope was licked, and 3) extracting the swab using an organic extraction and purification method of Phenol, Chloroform, and Isoamyl alcohol, followed by n-Butanol and an ultra-4 column One benefit of targeting mitochondrial DNA (mtDNA) in this study is that it offers a direct correlation with mtDNA profiles from skeletal remains often analyzed at AFDIL. Due to the sensitive nature of mtDNA analysis, the steam bath method can yield inconclusive results and introduce contamination from individuals who have previously handled the envelope.

Here, a new method was developed that maximizes the quantity of authentic DNA recovered from envelopes. To mimic casework conditions, mock envelopes containing a known mtDNA sequence in the seal of an envelope and a different known mtDNA sequence on the outside were created for experimentation. These mock envelopes were used to evaluate how the following variables affected nuclear and mitochondrial DNA yields: 1) the amount of extraction buffer and proteinase K volumes, 2) the type of sample collection (steam bath-swabbing vs. cutting or punching), and 3) the type of extraction: organic vs. QIAamp DNA Investigator Kit™ on the QIAcube™ by QIAGEN®. Nuclear DNA yield was assessed by Quantifiler™ Duo and mtDNA quality was assessed by relative fluorescence units (RFU) observed in mtDNA sequence data.

The results indicate that increasing the volume of extraction buffer from 500 µl to 1250

µl and proteinase K from 20 µl to 50 µl increases the quantity of DNA obtained. Secondly, a cutting of the seal, rather than a swabbing, yields higher quantities of both nuclear and mtDNA after an organic extraction. A comparison with the QIAamp DNA Investigator kit™ extraction protocol performed on an automated QIAcube™ instrument revealed that the organic extraction method resulted in higher quantities of DNA. Although mock envelopes were purposefully created to contain a mixture, none were observed. This is likely due to a non-shedder being used for contamination and the more recent deposition of buccal swabs in the seal of the mock envelopes compared to historic envelopes.

Continued evaluation of the method will incorporate heirloom envelopes similar to those encountered by the AFDIL casework sections. Additionally, the effect of UV irradiation on the heirloom envelopes will be assessed to minimize the levels of exogenous DNA while ideally not causing significant harm to the DNA contained within the seal.

Introduction

A robust DNA extraction method optimizes the quantity of DNA recovered for use in downstream analyses and is, therefore, essential to producing full-profiles for human identification. At AFDIL, the currently validated DNA extraction protocol for envelopes and stamps gives consistently poor results, resulting in no amplifiable mtDNA or mixtures. Consequently, the use of envelopes as alternative reference samples to assist in the identification of fallen soldiers is less desirable, despite being a commonly available source of DNA. The need for an overhaul of the extraction protocol is necessary for AFDIL's ability to continue its work in the identification of soldiers when there are no direct family members to provide a reference DNA sample.

Currently, the envelope extraction protocol uses a steam bath and tweezers to open the seal of an envelope followed by a dime-sized swabbing and organic extraction (1). The leading problem with this approach is that the steam bath saturates the paper of the envelope long before the seal of the envelope is softened, causing the paper to rip apart during the seal separation process. Additionally, it is believed that the steam bath in combination with tweezers is a source of the false-positive mixtures that are observed in mtDNA sequence data. Because the envelope is saturated with water, the envelope contributor's DNA on the outside of the envelope might be mixed with the authentic DNA within the seal from capillary action, and if the tweezers are not used in the same orientation at all times, the DNA can be transferred from the outside of the envelope to the seal on the inside. Another issue with the current protocol is the low quantity of DNA recovered when the steam bath is successful in separating the two sides of the envelope seal and has been reported earlier and attributed to the multiple centrifugation and tube transfer steps in the organic extraction procedure (11). More often than not, the quantity of DNA obtained from envelopes at AFDIL is too low to be properly sequenced.

Mitochondrial DNA analysis is used in this study due to the nature of AFDIL's mission: identifying the remains of fallen service members from current and past conflicts. Aged or degraded remains from WWII and the Korean War, for example, may not have enough well preserved nuclear DNA for identification. When a sample does have enough nuclear DNA, it usually results in a partial profile that is unsuitable for identification (8). Since mtDNA is found in much higher quantities in human cells, and because it can be amplified and sequenced in small segments, mtDNA is often the only means of DNA typing from aged or skeletonized remains. MtDNA is also a nonrecombining, maternally inherited genome, which makes it ideal for direct comparison in cases of distantly related relatives. AFDIL therefore collects DNA reference

samples from living relatives of all missing soldiers, and mtDNA is critical to the identification process.

When DNA samples are not available from maternally related family members, other reference samples must be used to make an identification from DNA. Envelopes written by fallen service members are therefore submitted for DNA analysis, and AFDIL strives to return the envelopes in as good condition as possible. In order to improve the currently validated envelope DNA extraction protocol, several modifications were evaluated. These include an increase in volume of both extraction buffer and proteinase K during the incubation period, which has been shown to increase total DNA extracted from a substrate (13); changing the sampling to a cutting and investigating how large a sample size is needed; using UV irradiation to limit the chance of contaminating a sample with DNA from the envelope contributor; and the use of the QIAamp DNA Investigator kit™ (QIAGEN, Hilden, Germany) to allow for an automated DNA extraction on the QIAcube™ (QIAGEN, Hilden, Germany);. The evaluation of the automated DNA extraction reflects the needs of the forensics community in shifting more and more toward automation in all aspects of DNA analysis (12).

Methods and Materials

Sample Description

In order to test the effective of each change in the current protocol, mock envelopes were created to mimic casework conditions. These mock envelopes were created by having Individual 1 lick and seal the envelope to leave their DNA within the seal, Individual 2 handling the envelope to leave their DNA on the outside of the seal, and Individual 3 processing the envelope for DNA analysis. The known mtDNA profile of each individual was used to determine which

regions of mtDNA would be examined to assess the results. Individual 1 has a T to C polymorphism at position16126 and a G to A polymorphism at position16390, while Individual 2 has an A to G polymorphism at position 16240, and Individual 3 has a C to T polymorphism at position16069.

Sample Collection

Samples were swabbed, cut or punched, immersed in varying volumes of extraction buffer and Proteinase K in a 2mL SpinEze™ tube (Fitzco Inc., Spring Park MN), and incubated at 56°C overnight. The primary focus of the experiments dealt with changes to the sampling method. The extraction negative control (reagent blank) consisted of varying volumes of extraction buffer (10mM Tris, pH 8.0, 100mM NaCl, 50mM EDTA, pH 8.0, 0.5% SDS) and proteinase K dependent on each experiment. The substrate blank for each experiment consisted of a swabbing of a supposedly DNA-free envelope. Table 1 reflects the various modifications that were tested.

Table 1 – Extraction protocol variations

	Sample Collection	Sample Size	Buffer Volume	UV Irradiation	Extraction Type
Envelope 1	Steam bath and Swabbing	Dime-sized swabbing, tip of swab	500 µL Extraction buffer, 20 µL Proteinase K	None	Organic
Envelope 2	Steam bath and Swabbing	Dime-sized swabbing, tip of swab	1.25 mL Extraction buffer, 50 µL Proteinase K	None	Organic
Envelope 3/4	Cutting	1 cm2	1.25mL Extraction buffer, 50 µL Proteinase K	5 minutes	Organic
Envelope 5	Cutting	1 cm2	1.25 mL Extraction buffer, 50 µL Proteinase K	0-9 minutes	Organic
Envelope 6	Cutting	1 cm2	1.25 mL QIAamp Digestion buffer	None	QIAamp DNA Investigator kit
Envelope 7	Cutting and Punching	6 sizes ranging $1cm^2$ to $0.08cm^2$	1.25 mL Extraction buffer, 50 µL Proteinase K	None	Organic

DNA Extraction

After incubating, the samples underwent an organic extraction per AFDIL's standard operating procedure (SOP). This process consists of piggybacking the substrate in a SpinEze™ spin basket and spinning for 3 minutes at 18,400g. The spin basket is removed and discarded. 500µL of Phenol, Chloroform, Isoamyl Alcohol (PCI) are added to the tube and then placed in a microcentrifuge at 18,400g for 2 minutes. The top layer of liquid in the tube is then transferred to a new sterile 1.7mL tube and the bottom layer of PCI is discarded. The PCI wash is performed twice. Next, 500µL of N-Butanol are added to the sample and spun at 18,400g for 2 minutes. The top layer of Butanol is discarded and the bottom layer is placed in a Microcon YM-30™ (Sigma-Aldrich Corp., St. Louis MO) spin basket and tube. This tube is spun at 18,400 g for 8 minutes, and the eluent is discarded. The basket is then filled with 40µL of TE^{-4} buffer (10mM Tris, 0.1mM EDTA, pH7.5), inversed, and placed in a new Microcon YM-30™ tube. This tube was then spun for 2 minutes at 1,320g. 60µL of additional TE^{-4} buffer were then added to the tube for a final volume of roughly 100µL.

Quantitative PCR (qPCR)

Aliquots of 5µL from the extraction were used to determine the concentration of nuclear DNA in each sample using Applied Biosystems® Quantifiler® Duo Human Quantification kit (Life Technologies™, Foster City, CA).

PCR Amplification

Samples were amplified using AFDIL's mini primer sets, MPS, 1A and 2B. Each MPS was used to evaluate results for mixtures and polymorphisms present. The master mix for a MPS amplification consists of: 5 µL 10X PCR buffer, 4 µL dNTPs, 2 µL Forward Primer, 2 µL

Reverse Primer, 1 µL AmpliTaq Gold® (Life Technologies™, Foster City, CA), 2 µL Non-Acetylated-BSA, 24 µL deionized water, and sample DNA. MPS1A contains the primers F15989 and R16158, while MPS2B contains F16222 and R16410-m19. MPS1A was placed on a GeneAmp 9700 thermal cycler (Life Technologies™, Foster City, CA) and amplified using the following program: 96° C for 10 minutes to activate the *Taq*, and then 38 cycles of 94° C for 20 seconds to denature, 50° C for 30 seconds to anneal, and 72° C for 30 seconds to extend. MPS2B was placed on a thermal cycle of 96° C for 10 minutes to activate the *Taq*, and then 42 cycles of 94° C for 20 seconds to denature, 48° C for 30 seconds to anneal, and 72° C for 30 seconds for extension. The final products were held at 4° C. These parameters are based on AFDIL's mtDNA amplification SOP. The amplification positive control consisted of 2µL HL60 Positive Human Control DNA (200 pg/µL) and 8µL deionized water, and the amplification negative control was 10µL of deionized water.

Yield Gel

Amplified mtDNA was first evaluated using a yield gel. FlashGel™ (Lonza Group, Basel, Switzerland) were used by first placing 4µL of water in each well, and then 5µL of sample DNA mixed with loading dye. A 50-1500 bp DNA ladder was placed in the wells encompassing the samples. The gel was run at 250V for roughly 3 minutes. After successful amplification was observed, the mtDNA went through the process of sequencing.

Post-PCR Purification and Sequencing

First, each sample was purified using 1.5 µL ExoSAP-IT® (Affymetrix Inc., Santa Clara, CA) and 18.5 µL SAP dilution buffer which degrades unused primers and dNTPs that remain in the amplification tube. Samples were placed on a thermal cycle of 30 minutes at 37° C to activate

the ExoSAP-IT® and 15 minutes at 85° C to inactivate the enzymes. Next, the samples underwent Sanger sequencing to incorporate the labeled bases into the numerous strands of mtDNA. In this case, samples for MPS1A and MPS2B were sequenced with 1 µL of either the forward or reverse primer of the appropriate mini primer set, 3.6 µL BigDye® Terminator v1.1 (Life Technologies™, Foster City, CA), 0.4µL dGTP BigDye® (Life Technologies™, Foster City, CA), 4.0 µL of Sequencing Dilution Buffer, 2 µL of sample DNA, and 9 µL of deionized water for a total volume of 20 µL. Samples are sealed in a 96-well tray and placed in a thermal cycle of 25 cycles consisting of 96° C for 15 seconds to denature, 50° C for 5 seconds to anneal, and 60° C for 2 minutes to extend. The product was held at 4° C.

Post-Sequencing Reaction Purification

After sequencing, an EDGE Biosystems Performa DTR 96-well Ultra Gel Plate Kit (EdgeBio, Gaithersburg, MD) was used to once again remove unused reagents from the samples. The EDGE block is first spun at 850 rpm for 5 minutes to remove excess water from the wells. Then the samples are placed in corresponding wells of the EDGE block and a new 96-well tray is placed under the block to collect the samples that run through. The EDGE block and 96-well tray are spun again at 850 rpm for 5 minutes. The 96-well tray containing samples was placed in a SpeedVac on high setting for 45 minutes in order to evaporate the liquid. The wells were then resuspended with 10µL Hi-Di Formamide.

Capillary Electrophoresis

The Applied Biosystems® 3130*xl* Genetic Analyzer (Life Technologies™, Foster City, CA) was used to detect the amplified and sequenced product. The samples were injected at 18 kV with a 5 second injection time and separated in Performance Optimized Polymer-6 (POP-6).

Data Analysis

All quantification results were compiled into a spreadsheet detailing the amounts of human and human male DNA that were detected in each sample.

All data was analyzed using Sequencher v4.0.5b11 (Gene Codes Corp., Ann Arbor, MI). Sequences were edited and aligned to the revised Cambridge Reference Sequence (rCRS) (9, 10).

Additionally, the quantification results were analyzed using an ANOVA test, followed by a Tukey post-hoc analysis of the results. The ANOVA values were calculated using an online calculator which gave the proper F-value and p- value. With the results of the ANOVA, a Tukey post-hoc analysis was performed using the following equation and critical value in order to determine which of the mean quantification values obtained from each experiment were statistically significantly different from one another (14,15).

Equation 1: $\frac{M_1 - M_2}{\sqrt{MS_w\left(\frac{1}{n}\right)}}$ Critical Value = 4.35

Results and Discussion

Casework SOP for Envelope Extraction – Envelope 1

The current protocol for envelope extractions was employed and produced the expected results. The quantification values for the samples showed only two samples with detectable amounts of human DNA. Those two, Env1-A and Env1-D, showed less than 10 pg/µL. Despite the low quantification results, the samples were sent through the entire workflow to see if the mtDNA in the samples was enough to generate a profile. The yield gel was blank for all of the samples, and after sequencing, no mtDNA profiles were generated.

Table 2 – Casework SOP for Envelope Extraction – Envelope 1.
The quantification results for Envelope 1. UND = Undetermined

	QuantDuo Results	
Sample Name	Duo Human (pg/µL)	Duo Male (pg/µL)
Env1-A	2.0	4.6
Env1-B	UND	7.9
Env1-C	UND	UND
Env1-D	8.1	10.9
Env1-SB	UND	UND
Env1-RB	UND	UND

Increased Lysis Buffer and Proteinase K – Envelope 2

For Envelope 2, the volume of extraction buffer and proteinase K were both increased by a factor of 2.5. The quantification data in Table 2 shows three of the four samples containing human DNA at concentrations ranging from 1.2 pg/µL to 11 pg/µL. Despite the still very low levels of nuclear DNA detected, the samples were amplified and sequenced. The yield gel indicated successful amplifications for all of the samples but not the reagent blank or substrate blank, and the sequence data showed polymorphisms 16126 C and 16390 A which are consistent with Individual 1. There was no indication of mixture in the samples and so Individual 2 and 3

were not present.

Table 3 – Increased Lysis Buffer and Proteinase K – Envelope 2.
Quantification results and observed polymorphisms in Envelope 2.

Samples	Duo Human (pg/µL)	Duo Male (pg/µL)	Observed Polymorphisms	
Env2-A	UND	UND	MPS1A Poly:	16126 C - Individual 1
			MPS2B Poly:	16390 A - Individual 1
Env2-B	11.5	UND	MPS1A Poly:	16126 C - Individual 1
			MPS2B Poly:	16390 A - Individual 1
Env2-C	2.4	1.6	MPS1A Poly:	16126 C - Individual 1
			MPS2B Poly:	16390 A - Individual 1
Env2-D	1.2	UND	MPS1A Poly:	16126 C - Individual 1
			MPS2B Poly:	16390 A - Individual 1
Env2-RB	UND	UND	MPS1A Poly:	No Data
			MPS2B Poly:	No Data
Env2-SB	UND	UND	MPS1A Poly:	No Data
			MPS2B Poly:	No Data

UV Irradiation to reduce mixtures – Envelopes 3 and 4

Envelopes 3 and 4, which each had one half exposed to UV irradiation, resulted in no observable mixture in the samples. However, by taking a cutting of the seal rather than swabbing the seal after a steam bath, the concentration of nuclear DNA observed in the samples ranged from 9.6 pg/µL to 130.6 pg/µL. The yield gel showed strong bands in samples A through D for both envelopes and a strong band for the Substrate Blank. After sequencing, the samples showed the desired polymorphisms at positions 16126 and 16390. Additionally, although no mixture was seen in the mock envelope samples, contamination was discovered in the substrate blank containing a mixture at eight positions. The mtDNA profile found in the substrate blank contained numerous polymorphisms, but they did not match the mtDNA profiles of Individuals 1, 2, or 3.

Table 4 – UV Irradiation to reduce mixtures – Envelopes 3 and 4
Quantification results and observed polymorphisms in Envelopes 3 and 4

Samples	Duo Human (pg/µL)	Duo Male (pg/µL)	Observed Polymorphisms	
Env3-A	71.7	44.5	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env3-B	37.2	85.5	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env3-C	130.6	207.0	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env3-D	34.1	46.7	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env4-A	59.3	96.5	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env4-B	55.1	43.1	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env4-C	92.9	138.5	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env4-D	9.6	UND	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env3-RB	UND	UND	MPS1A Poly:	UND
			MPS2B Poly:	UND
Env3-SB	UND	UND	MPS1A Poly:	16090 T, 16106 A, 16129 A
			MPS2B Poly:	16278 T, 16294 T, 16309 A, 16368 T, 16390 A

UV Irradiation time required to reduce mixtures – Envelope 5

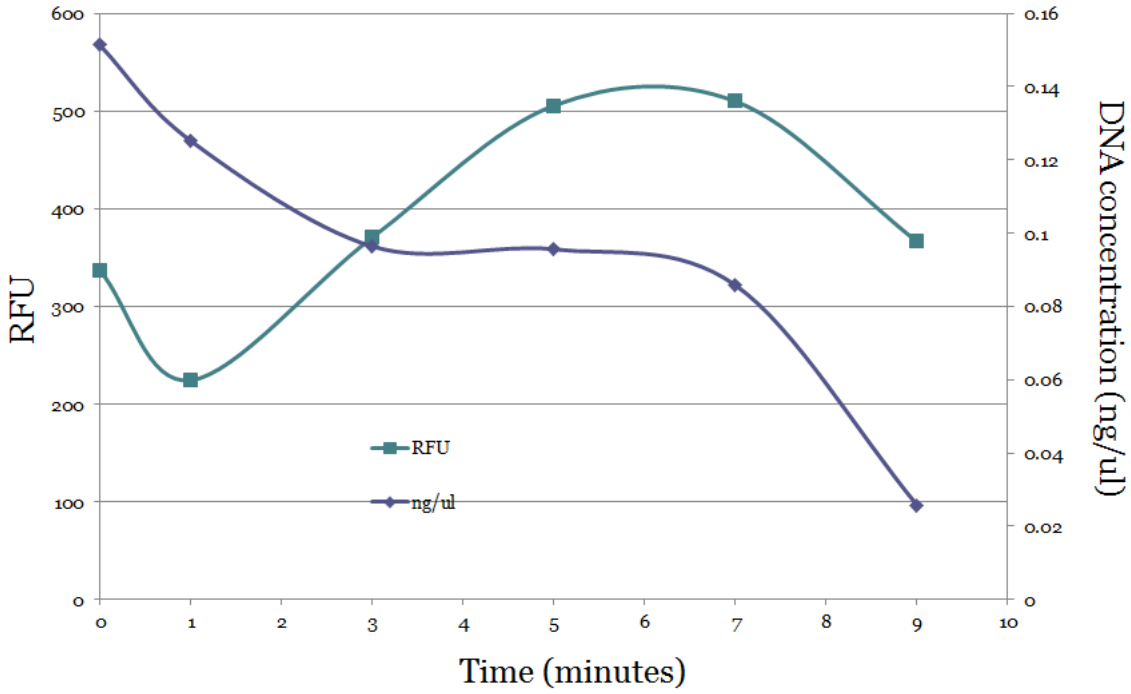
Envelope 5, which consisted of 6 samples receiving increasing amounts of UV irradiation, had relatively large amounts of nuclear DNA recovered from the envelope. The DNA concentration ranged from 25.8 pg/µL to 151.3 pg/µL. The sample that received the highest amount of UV irradiation, Env5-F, had the lowest amount of DNA recovered, while the sample with no UV irradiation, Env5-A, had the highest amount of DNA recovered. The yield gel for Envelope 5 showed strong bands for samples A through F and a very weak band for the Reagent Blank. Samples A through F all contained the polymorphisms of Individual 1 and no mixtures were observed. The reagent blank, Env5-RB, however contained two polymorphisms. These two

polymorphisms are not consistent with Individuals 1, 2, or 3. The average Relative Fluorescence Units (RFU), of each sample was taken and compared with the quantification results in order to observe the correlation between DNA concentration and signal strength of the final product. Although samples D through F had much larger RFU than samples A and B, it does appear that the continued exposure to UV irradiation causes the intended effect of diminishing total DNA concentration from the extraction process and to all downstream analysis.

Table 5 – UV Irradiation time required to reduce mixtures – Envelope 5
Quantification results and the polymorphisms observed in Envelope 5 samples.

	Duo Human (pg/µL)	Duo Male (pg/µL)	Observed Polymorphisms	
Env5-A	151.3	111.3	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env5-B	125.2	162.5	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env5-C	96.4	82.0	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env5-D	95.6	38.4	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env5-E	85.8	57.6	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env5-F	25.8	34.2	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env5-RB	UND	UND	MPS1A Poly:	No Data
			MPS2B Poly:	16292 T, 16362 C
En5-SB	UND	UND	MPS1A Poly:	No Data
			MPS2B Poly:	No Data

Figure 1 – UV Irradation time required to reduce mixtures – Envelope 5
The average RFU compared to DNA concentration as UV time increases



Automation of Envelope Extraction – Envelope 6

The quantification results of Envelope 6 showed an overall lower amount of DNA recovered than the organic extraction method. Env6-B however, resulted in having the highest concentration of DNA of any sample thus far. The yield gel for Envelope 6 showed strong bands for samples A through D and the Reagent Blank and Substrate Blank showed no band. The resulting profiles contained the polymorphisms at positions 16126 and 16390 that are consistent with Individual 1. There was no indication of a mixture in the samples at any point.

Table 6 – Automation of Envelope Extraction – Envelope 6.
The quantification results and observable polymorphisms for Envelope 6.

Samples	Duo Human (pg/µL)	Duo Male (pg/µL)		
Env6-A	18.1	40.3	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env6-B	218.6	205.1	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env6-C	26.3	24.5	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env6-D	10.4	17.5	MPS1A Poly:	16126 C – Individual 1
			MPS2B Poly:	16390 A – Individual 1
Env6-RB	UND	UND	MPS1A Poly:	No Data
			MPS2B Poly:	No Data
Env6-SB	UND	UND	MPS1A Poly:	No Data
			MPS2B Poly:	No Data

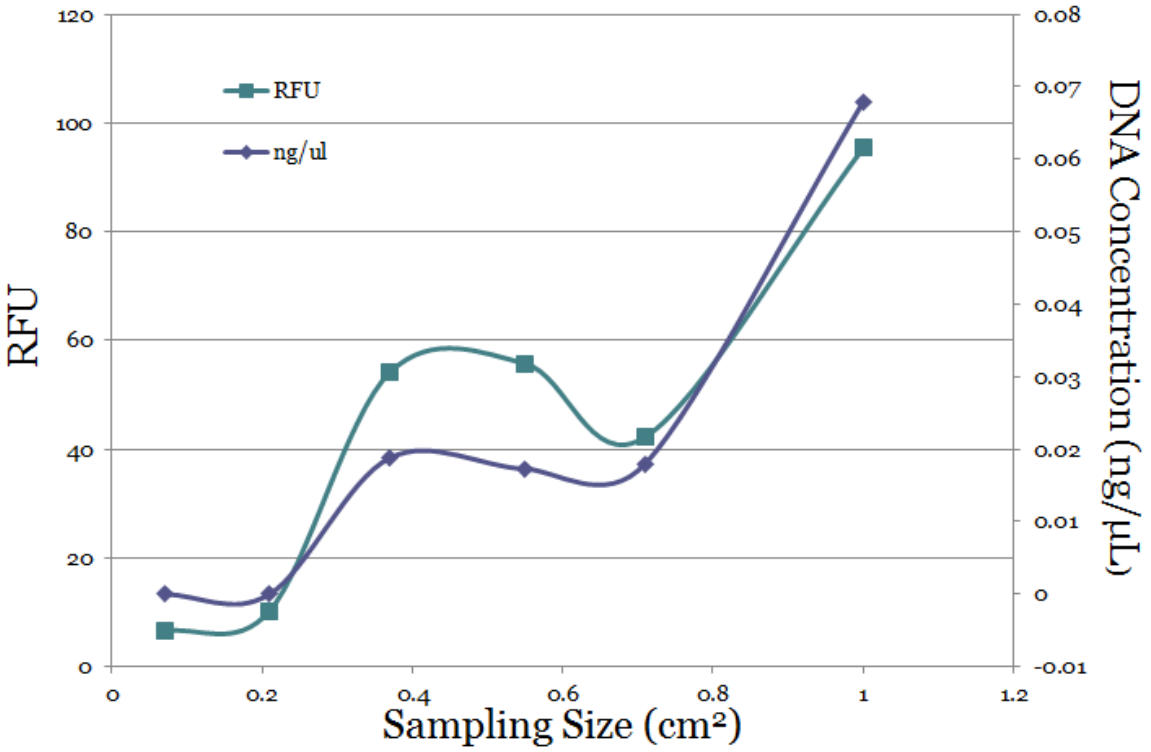
Sampling Size for Envelope Extraction – Envelope 7

Envelope 7, a gradually decreasing sample size experiment, produced the intended quantification and RFU results. The quantification results show the intended decrease in DNA recovered with decreased sample size. And as can be seen in Figure 2, The RFU of each sample falls in an almost identical manner. In Table 6, the results for MPS1A are missing due to the samples not amplifying at all, despite a successful amplification of the positive control. The samples were amplified on three separate occasions with different reagents, but only the amplification positive control showed successful amplification. Despite missing the results of one mini primer set, MPS2B still showed no suggestion of a mixture and the correct polymorphisms were identified.

Table 7 – Sampling Size for Envelope Extraction – Envelope 7.
The quantification and observed polymorphisms listed for Envelope 7.

Samples	Duo Human (pg/µL)	Duo Male (pg/µL)		
Env7-A	67.9	60.8	MPS1A Poly:	N/A
			MPS2B Poly:	16390 A – Individual 1
Env7-B	18.0	16.8	MPS1A Poly:	N/A
			MPS2B Poly:	16390 A – Individual 1
Env7-C	17.3	17.2	MPS1A Poly:	N/A
			MPS2B Poly:	16390 A – Individual 1
Env7-D	18.8	22.2	MPS1A Poly:	N/A
			MPS2B Poly:	16390 A – Individual 1
Env7-E	UND	UND	MPS1A Poly:	N/A
			MPS2B Poly:	No Data
Env7-F	UND	1.6	MPS1A Poly:	N/A
			MPS2B Poly:	No Data
Env7-RB	UND	UND	MPS1A Poly:	N/A
			MPS2B Poly:	No Data
Env7-SB	UND	UND	MPS1A Poly:	N/A
			MPS2B Poly:	No Data

Figure 2 – Sampling Size for Envelope Extraction – Envelope 7
The average RFU compared to DNA concentration as sampling size



Statistical Analysis

ANOVA and a Tukey post-hoc analysis were used to determine the statistical significance of the Quant Duo results obtained from each experimental condition. Table 8 shows the mean and standard deviation for each envelope. Figure 3 shows the results of the ANOVA showing the F-value of 3.766, and p-value of .011; therefore the differing Quant Duo results obtained from the differing experimental conditions were statistically significant. Table 9 shows the values for the Tukey post-hoc analysis of the ANOVA values.

Table 8 – Statistical Analysis of Envelopes
The mean and standard deviation of each envelope group

	Mean	Std. Dev.
Envelope 1	0.002525	0.003834
Envelope 2	0.003775	0.005242
Envelope 3/4	0.0613	0.037702
Envelope 5	0.096683	0.042286
Envelope 6	0.06835	0.100377
Envelope 7	0.020333	0.024926

Figure 3 – Statistical Analysis of Envelopes
ANOVA results of envelopes

	SS	df	MS	F	p
Between:	0.038	5	0.008	3.766	0.011
Within:	0.052	26	0.002		
Total:	0.09	31		———	

Table 9 – Statistical Analysis of Envelopes
Tukey post-hoc results of each envelope compared to the others

	Compared to	Tukey Post Hoc value
Envelope 1	Envelope 2	-0.064332964
	Envelope 34	-3.02493596
	Envelope 5	-4.843654581
	Envelope 6	-3.387773876
	Envelope 7	-0.916513136
Envelope 2	Envelope 34	-2.960602996
	Envelope 5	-4.779321618
	Envelope 6	-3.323440912
	Envelope 7	-0.852180172
Envelope 34	Envelope 5	-1.821034608
	Envelope 6	-0.362837916
	Envelope 7	2.108422824
Envelope 5	Envelope 6	1.458196692
	Envelope 7	3.929457432
Envelope 6	Envelope 7	2.47126074

With a combination of minor adjustments to the current protocol of DNA extraction from envelopes, the results obtained from mtDNA analysis were greatly improved. The increased volumes of extraction buffer and proteinase K in Envelope 2 showed a statistically insignificant increase in DNA recovered, however that procedure still suffered from the use of a steam bath and swabbing collection method. Envelopes 3 and 4, which were the first envelopes to use a $1cm^2$ cutting rather than swabbing, showed vastly improved results. Envelopes 3 and 4 were also meant to demonstrate the use of UV irradiation for the reduction of mixtures, however no mixtures were ever found in either the non-UV treated or UV treated samples. It should be noted however, that the DNA within the seal of the envelope did not suffer greatly from the UV irradiation. As seen in Envelope 5, after seven minutes of UV irradiation noticeable amounts of DNA were still present in the seal of the envelope and successful amplification was achieved using the mini primer sets.

Automation of the overall process in order to save time and energy on behalf of the analyst, and to lower the chances of human error, did not match the results of the organic extraction. This is consistent with prior research into the use of the QIAcube™ (16), and helped to determine that the organic extraction is the most efficient procedure for the extraction of DNA.

The final experiment demonstrated the need for an adequate sample size in the course of the extraction process. Although samples as low as $0.4cm^2$ still showed adequate results, taking a $1cm^2$ guaranteed the highest amount of DNA recovered. Although a least destructive form of sampling is desired throughout all of DNA analysis, it is still necessary to make sure that a sample is going to give the best results possible on the first try. Additionally, throughout all the envelopes, it was observed that the middle third of the seal consistently contained higher levels of DNA compared to the outer thirds of the envelope.

There was a statistically significant difference between groups as determined by one-way ANOVA ($F(5,26) = 3.766$, $p = .011$). Using a Q table for Tukey's HSD, the critical Q(.05) value, given 26 degrees of freedom and six treatments per group, is 4.35. A Tukey post-hoc test revealed that, the quantification results of Envelope 5 are statistically significantly different from Envelope 1, Envelope 2, and Envelope 7. This suggests that Envelope 5's larger sample size and increased buffer volumes lead to a definite increase in total DNA recovered from a sample.

Conclusion

Overall, the process of DNA extraction from envelopes at AFDIL would greatly improve with just a few changes. First, the steam bath and swabbing should be abandoned in favor of using a 1cm2 cutting from the middle third of the envelope. Next, the volumes of extraction

buffer and proteinase K should be increased to 1250 and 50 µL respectively. Automation of the process, as opposed to Organic extraction is not suitable at this point in time. Finally, if there is any suspicion of a mixture, the envelope should be placed in a cross linker for UV irradiation for no more than seven minutes. With these adjustments put in effect, there is no doubt that DNA analysis from envelopes for the identification of fallen service members would become a much more viable strategy.

Future study would call for testing the method on heirloom envelopes, as well as attempting to ensure that a shedder is used to replicate mixtures. The Quality Control section of AFDIL could also reproduce these results on heirloom envelopes in order to validate the improved protocol and put it in to use for casework.

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